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New and Modified Techniques for Studying Nitrogen-Fixing Bacteria in Small Mammal Droppings

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Abstract

Nitrogen-fixing bacteria in small mammal droppings are potentially important to forest productivity. As we study this phenomenon, however, we continually find unknowns, such as bacteria that we cannot isolate and purify because we do not know which techniques to use. For example, we have recently observed acetylene reduction in the droppings of the tundra vole (*Microtus oeconomus*) from St. Lawrence Island, Alaska, but we have failed three times to isolate the responsible bacterium. We hope this note will help stimulate parallel research on use of techniques under various circumstances.

Keywords: Laboratory methods, nitrogen-fixing bacteria, mammals (land).

Collecting Fecal Samples

We devised four alternative methods for collecting fecal samples—methods that ensured as much sterility as possible.

1. Catch the animal in a trap that keeps it alive and unharmed. Remove it from the trap and place it into a clean, sterilized cloth bag. Leave the animal in the bag for three to five minutes to defecate then release it. Pick up the deposited fecal pellets with sterile forceps and place the pellets in a sterile vial or petri dish.

2. Catch the animal in a live trap. Remove it from the trap and, while holding it upside down, gently remove any exposed fecal pellets with sterile forceps and place the pellets in a sterile vial or petri dish.

3. Catch the animal in a killing trap, excise the terminal fecal pellets, and place them into sterile receptacles with sterile forceps.

4. Catch the animal and put it into a thoroughly clean and, if possible, sterilized cage. Collect the fecal pellets with sterile forceps as the animal deposits them.

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Laboratory Techniques

We used the fecal pellets of three small mammals: (1) California red-backed vole (*Clethrionomys californicus*), (2) northern flying squirrel (*Glaucomys sabrinus*), and (3) deer mouse (*Peromyscus maniculatus*). To test for the presence of nitrogen-fixing bacteria, as measured by the acetylene reduction assay, Döbereiner nitrogen-free liquid medium (Döbereiner and Day 1976) was used for feces of the California red-backed vole and the northern flying squirrel. It worked well for these rodents because the nitrogen-fixing bacterium turned out to be *Azospirillum* sp. This medium did not work for the deer mouse pellets, however, so we tried Burk's nitrogen-free liquid medium (Burk 1930). Burk's medium worked well because the nitrogen-fixing bacterium in the deer mouse feces was *Clostridium butyricum*.

One or two fecal pellets were placed in 20 mL of nitrogen-free liquid medium in a 60-mL capacity serum bottle. Bottles were capped and flushed for 5 min with nitrogen gas containing less than 10 p/m oxygen. The liquid medium became turbid after incubation for 2 days at 30 °C. Acetylene was then injected into each bottle to 10 percent (v/v); the bottles were gently swirled immediately after addition of acetylene and left to stand at 30 °C. Bottles without acetylene injection served as controls. After 18 hr, 0.1-mL gaseous samples from each bottle were removed and analyzed for ethylene and acetylene with a Hewlett-Packard 5830A gas chromatograph[1/] fitted with a 2-m × 2.1-mm, 80-100 mesh, Porapak R column. Oven temperature was adjusted to 70 °C. Injection and flame ionization detector temperatures were each adjusted to 100 °C. Nitrogen carrier gas flow rate was adjusted to 40 $mL^{-1} \cdot min^{-1}$.

Bacterial cultures that reduced acetylene were isolated in two ways: (1) by repeatedly streaking the cultures in the bottles on Döbereiner agar medium containing 0.002 percent yeast extract, and (2) by streaking and incubating the Döbereiner and Burk agar media at 30 °C in an anaerobic chamber in which generator-released hydrogen gas combined with atmospheric oxygen in the presence of a catalyst to form water. The result was an anaerobic atmosphere in the chamber that contained approximately 5-9 percent carbon dioxide throughout the incubation period. (The anaerobic system we used is produced by: BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD USA.)

Clostridium butyricum, isolated from the feces of the deer mouse, was tested for its ability to reduce acetylene to ethylene by a method slightly modified from the Pankhurst tubes as described by Campbell and Evans (1969). The two H-tube arms (63 mL total volume) were identical in size and attached to each other by a connecting tube fitted with a ground glass joint and a clamp. One arm of each H-tube contained 10 mL of Burk's liquid medium supplemented with 0.002 percent yeast extract. After sterilization of the tubes with the medium in one arm and non-absorbant cotton in each connecting tube, sterile stoppers were inserted into each arm and the tubes flushed with sterile nitrogen gas. Alkaline Pyrogallol (2.0 mL) was injected into the arm not containing the liquid medium. Bacterial suspension (0.01 mL) was inoculated into the medium with a sterile syringe and needle. Control tubes were inoculated with sterile distilled water.

[1/]Use of a trade name does not imply endorsement or approval of any product by the USDA Forest Service to the exclusion of others that may be suitable.

For *Azospirillum* sp., isolated from the feces of the red-backed vole and the flying squirrel, a suspension (0.01 mL) in sterile distilled water was added to serum bottles that contained 20 mL Döbereiner's nitrogen-free liquid medium supplemented with 0.002 percent yeast extract. The bottles were capped with sterile serum stopper closures and flushed with nitrogen for 5 min. Oxygen was added to 1 percent of the gas volume. Cultures in the H-tubes and serum bottles were incubated at 30 °C for 3 days. Acetylene was injected into each H-tube and bottle, and the aforementioned procedure of ethylene determination followed.

To express the nitrogenase activity per unit of bacterial protein, bacterial cells were harvested and washed with cold 5 percent trichloroacetic acid. Protein was solubilized in 0.5 N sodium hydroxide in boiling water for 10 min (Agarwal and Keister 1983) and measured by the modified Lowry method (Markwell et al. 1978).

Yeast populations in feces were determined by the dilution plate method on sodium albumenate agar (Waksman and Fred 1922).

Conclusion

The best method for excising rodent fecal samples is under sterile conditions from a freshly killed animal. This does necessitate killing, however, which is probably not essential except under particularly stringent laboratory research design. We found holding animals in sterile cloth bags until they defecated to be preferable under most conditions. Further, a particular individual can be sampled repeatedly.

Nitrogen-free liquid media can be used to test for the presence of nitrogen-fixing bacteria in the feces of forest rodents. Place one or two fecal pellets in the liquid medium, flush for 5 min with nitrogen gas containing less than 10 p/m oxygen, and incubate at 30 °C. The resulting turbid liquid can be tested for nitrogenase activity by its ability to reduce acetylene to ethylene.

Döbereiner nitrogen-free medium, supplemented with yeast extract, is effective for *Azospirillum* sp. *Azospirillum* sp. can be isolated by repeatedly streaking the cultures on Döbereiner medium followed by incubation under anaerobic and aerobic conditions.

Clostridium butyricum can be isolated by incubating the streaking cultures on Burk's nitrogen-free medium, supplemented with yeast extract, under anaerobic conditions. This system is selective for *Clostridium butyricum*.

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English Equivalents

1 millimeter (mm) = 0.0394 inch
1 meter (m) = 39.37 inches
1 milliliter (mL) = 0.034 fluid ounces
°C = (°F −32)/1.8

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